Distribution of Rubber in Cryptostegia as a Factor in Its Recovery[1]

R. T. Whittenberger,[2] B. A. Brice,[2] and M. J. Copley[2]

ALTHOUGH *Cryptostegia* has long been known to contain rubber in quantity,[3] no extensive work on its commercial recovery had been done until the recent war emergency arose. In 1942 investigations on the recovery of rubber from *Cryptostegia* were begun in this laboratory and elsewhere.[4-9] The investigation in this laboratory dealt with the recovery of rubber from harvested stems and leaves and was not directly related to methods of recovery based on tapping or clipping twig ends of the growing plant.[4, 6, 7]

Early in the work difficulties were encountered, both in analytical and recovery procedures, of such a nature as to indicate that a substantial proportion of the rubber in the plant was not extractable by conventional methods expected to be applicable to plants having rubber as latex in a cell or duct system. Although analysis showed that most of the rubber in a two- or three-year-old plant occurred in the leaves, only a minor fraction of this rubber was recoverable by mechanical procedures, such as ball milling or crushing. Attempts to leach the rubber out of the leaves by immersion in latex-stabilizing solutions were also unsuccessful, as were all other treatments designed to recover the rubber as latex. In solvent extraction the initially recovered fraction had properties superior to those subsequently recovered. Finally, special chemical pretreatment was necessary for complete recovery by benzene extraction.

The present investigation was undertaken to ascertain the character and distribution of rubber in *Cryptostegia*, for the purpose of developing more satisfactory methods for its extraction. Heretofore it had been assumed that all the rubber in *Cryptostegia* occurred in a laticiferous duct system. This study has shown that although the relatively small quantity of rubber in stems is exclusively in the ducts, 85 to 90% of the rubber in mature *Cryptostegia* hybrid leaves occurs in individual chlorenchyma cells, and only 10 to 15% in the ducts. In addition the rubber-bearing *Cryptostegia* chlorenchyma cell has been found to differ from that of the rubber-bearing parenchyma cells of guayule[10] and pingue,[11] from which rubber is readily extracted by ball milling. Whereas the guayule cell contains rubber in the finely divided or latex state, there is no such latex in the *Cryptostegia* leaf chlorenchyma. These unexpected findings offer a full explanation for the difficulties encountered in the extraction of rubber from *Cryptostegia* leaves and should be of considerable interest to plant anatomists and physiologists.

The plants used throughout this investigation were principally *Cryptostegia* hybrid (*C. madagascariensis* Boj. x *C. grandiflora* R. Br.), grown in Florida and shipped under refrigeration to this laboratory. *Cryptostegia grandiflora* R. Br. also was used, some received from Cuba and Mexico, and some grown locally in a greenhouse.

Numerous microscopic observations were made on free-hand and microtone sections of both fresh and preserved stem and leaf tissues; these were supplemented by examination of unsectioned material under the dissecting microscope. Rubber was identified by staining with Calco Oil Blue Na.,[12] by solubility behavior, by micromanipulation, by X-ray diffraction studies, and by chemical analysis.[13]

Distribution of Rubber in Stems

Photomicrographs of stained sections of stems are shown in Figures 1 to 5. In the stems the rubber is found only in latex ducts; none has been observed in the cortex chlorenchyma. The ducts are scattered vertically throughout the pith and bark (phloem, phloem rays, and cortex), although a small number extend laterally in the wood rays and make possible an interchange of latex between the pith and bark. The ducts, occasionally branched and essentially without septa, range from five to 35 microns in diameter; the average is about 25 microns. In small stems (three to four millimeters in diameter) the pith ducts are larger and of more uniform size than those of the bark. The parenchyma cells adjoining the ducts frequently are gorged with starch grains, and some contain crystals (probably calcium oxalate).

The relative proportion of rubber in the bark and pith depends, of course, on the age, size, and development of the stem. Generally in stems of small diameter most of the rubber is in the pith; whereas in larger stems it is in the bark. An estimate based on the number of latex ducts, allowing for their variable size, indicates that in a young stem of three- to four-millimeter diameter approximately 75% of the total rubber occurs in the pith; the remaining 25% is present in the bark and, to a slight extent, in the wood. The pith of such a stem often contains from 300 to 400 latex ducts. Dissection and chemical analysis of three medium to large stems of seven-, nine-, and 17-millimeter diameter showed that 49, 64, and 74%, respectively, of the total rubber occurred in the bark. Viswanath *et al.*[9] report having recovered 0.96 and 0.07% rubber, air-dry basis, from the central pith and bark, respectively. No details, however, are given concerning the size of stems utilized, and it should be pointed out that the data were obtained by recovery rather than analytical procedures and indicate the concentration of rubber in tissues rather than the distribution of total rubber.

Distribution of Rubber in Leaves

Rubber is found in non-septate laticiferous ducts of the petiole and blade of the leaf. The ducts, present in approximately equal numbers in the tissue above and below the veins, parallel and branch with the veins. A cross-section of the midrib is shown in Figure 8, and typical vein patterns are shown in Figures 6 and 7. In a mature leaf the ducts are most numerous (50 to 100) and largest (10 to 25 microns in diameter) along the main vein; they decrease gradually in number and size with each decrease in size of the veins as they branch and ramify throughout the leaf blade. Associated with each of the 20 to 25 largest lateral veins are 10 to 20 ducts of about eight to 20 microns in diameter. Each of the smallest veins terminates with a single tracheid (Figure 7), and occasionally is devoid of ducts. A few of the smallest ducts may extend into the vein islets and into the non-vascular tissue at the periphery of the leaf. Individual leaves differ widely in the number of ducts and therefore in the amount of latex rubber they contain.

During the study of the leaf ducts, globules in the chlorenchyma cells were observed which behaved like rubber toward stains and organic solvents. Typical globules are shown in Figures 9, 10, 11, 12, 14, and 15; Figure 13 shows cells from which the globules have been removed by ether. The globules are found in all green cell types (palisade parenchyma, spongy parenchyma, guard cells) of the mature leaf, although they are most numerous (1 to 8 per cell) and largest in the palisade layer. Their general correlation with the presence of chlorophyll may be physiologically

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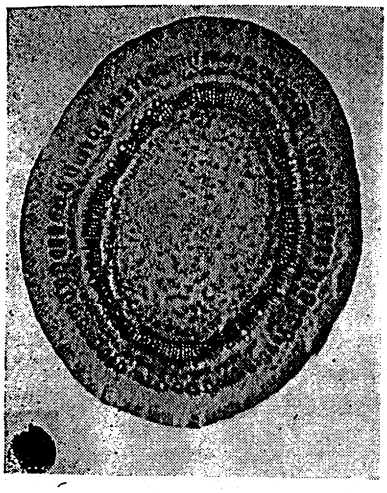


Fig. 1. Young Cryptostegia grandiflora stem, cross-section, 16X. Dark stained spots in pith and bark represent rubber in laticiferous ducts. Although much rubber inevitably was removed from the ducts during the preparation of the section, the distribution of rubber in a stem of small diameter (3 mm.) is indicated.

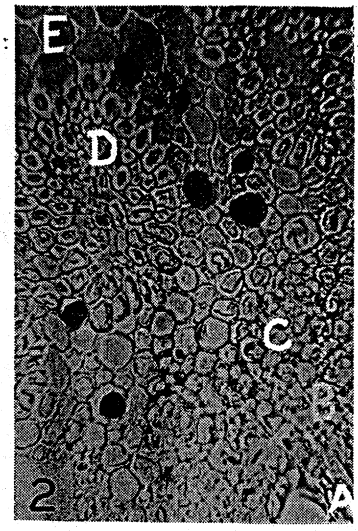


Fig. 2. Young Cryptostegia grandiflora stem, cross-section, 215X. Rubber is indicated by the dark stained areas. A, xylem; B, cambium; C, phloem; D, bast fibers; E, cortex.

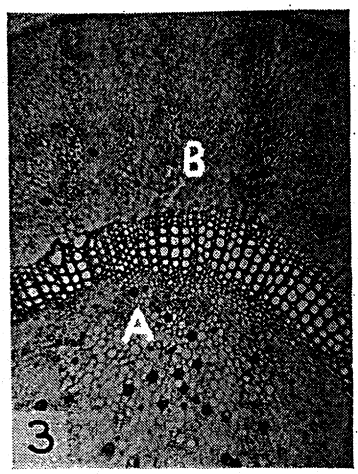


Fig. 3. Young Cryptostegia grandiflora stem, cross-section, 50X. Dark stained areas in pith (A) and bark (B) are rubber.

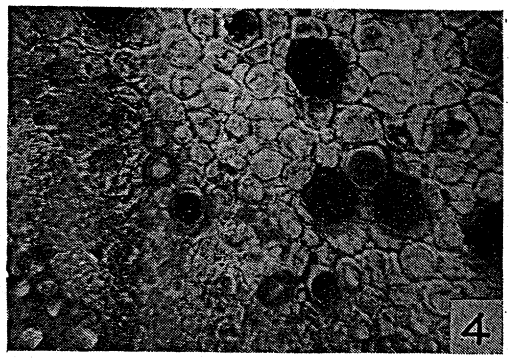


Fig. 4. Young Cryptostegia grandiflora stem, cross-section, 215X. Stained rubber in laticiferous ducts of the pith is shown. Xylem is seen at lower left.

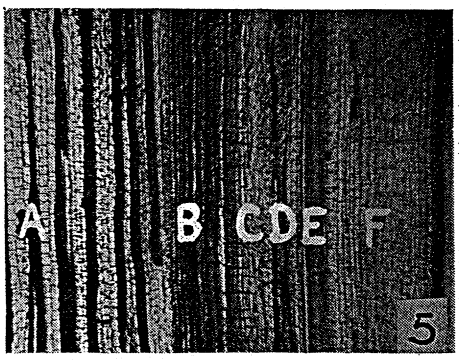


Fig. 5. Young Cryptostegia grandiflora stem, radial-longitudinal section, 50X. The extent of the laticiferous system is indicated by the stained rubber, although some rubber was removed and displaced during the preparation of the section. A, pith; B, xylem; C, cambium; D, phloem; E, bast fibers; F, cortex.

significant; the existence, in some cases at least, of very small globules in non-chlorophyllous parenchyma adjacent to the laticiferous ducts associated with the larger veins suggests a possible interchange of globular and duct material. Furthermore in the dorsal half of the leaf (spongy parenchyma) the globule-bearing cells are more numerous adjoining the ducts than in non-duct areas (Figure 14). Within the cell the larger globules are entirely distinct from the chloroplasts; whereas some of the smallest ones apparently are not. The globules may attain a diameter of 10 to 12 microns in the palisade cells, although smaller diameters (three to seven microns) are more common. It should be pointed out, however, that the globules vary greatly in size, frequency, and shape, depending upon the development, age, and previous history of the leaf. In young leaves only small globules, or none at all, are seen; in mature leaves of unusually high rubber content the chlorenchyma cells are heavily laden with globular material. In the latter cells many of the globules are distended to form oval or rod-shaped masses. These observations should be of value to physiologists in developing a theory of the mechanism of rubber formation in plants.

Identification of Rubber in the Globules of the Leaf Cells

The globules were clearly visible as pale yellow-green structureless masses, even in untreated sections of living mature leaves, as shown in Figure 9. These cell globules stained readily with Sudan III, alkanet, and Calco Oil Blue NA. A section bleached with Javelle water, extracted with acetone for 48 hours, and stained with Calco Oil Blue NA is shown in Figure 12. A similar section, bleached with Javelle water, extracted with ethyl ether for 24 hours, and stained, showed no globules (Figure 13). Similar observations with various solvents indicated that for the most part the globules were also soluble in benzene, toluene, xylene, carbon tetrachloride, and carbon disulphide. They were sparingly soluble in acetone, as shown by their decreased size after acetone extraction, and were only slightly affected by glacial acetic acid, Javelle water, chloral hydrate, and aqueous potassium hydroxide.

When subjected to tension by two micro-needles, a single globule stretched about tenfold before rupture. After rupture, the stretched threads quickly retracted, and two separate globules were formed. The globule, however, was tacky and stuck to the glass needle when punctured. These staining solubility, and micrurgical tests indicate that the globules contained rubber.

Further evidence on this point was obtained by chemical analysis of two carefully dissected fractions of the leaf, one essentially free of latex ducts and one essentially free of cells bearing the globules. From each of six *Cryptostegia* hybrid shoots three leaves were plucked, and the sample was divided into three lots. Only uninjured, non-chlorotic, turgid clean leaves were selected; some were young leaves near the apex; others were the older lateral leaves. No latex was lost from the leaves through exudation. They were infiltrated under suction successively with acetic acid, water, and dilute sodium hydroxide in order to coagulate any dispersed latex and to soften them sufficiently for dissection.

A complete separation of all the latex ducts from the remainder of the leaf was not attempted. However, since the quantity of latex duct rubber appeared to be closely correlated with the quantity of veins, it was believed that the removal of the main vein would remove a like proportion of the latex ducts. It was shown

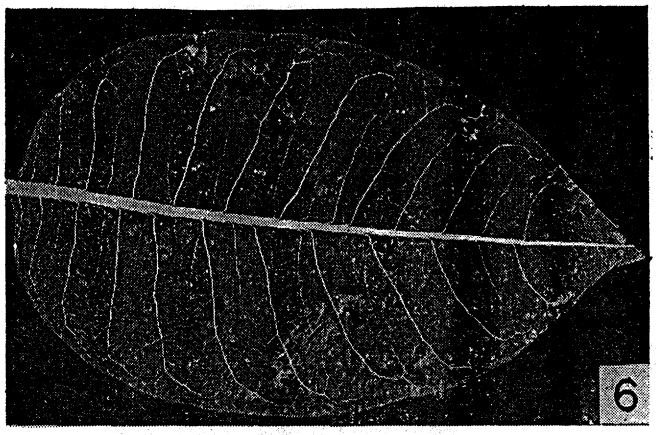


Fig. 6. Vein pattern of Cryptostegia hybrid leaf, 1X. The leaf was retted, and all tissue except the xylem of the veins was removed. The distribution of the xylem indicates the distribution of the laticiferous system. In a few areas the veins were damaged during preparation of the mount.

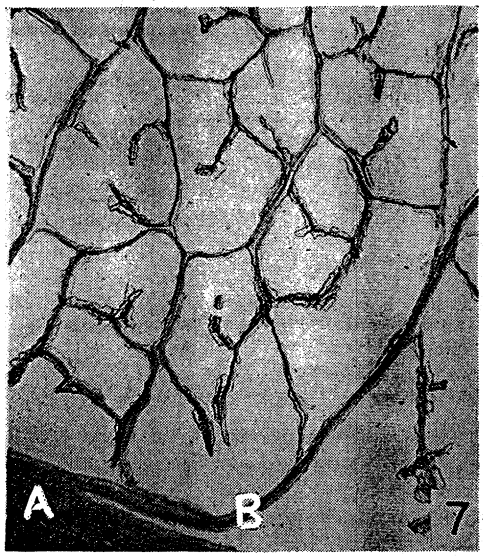


Fig. 7. Vein pattern of retted Cryptostegia hybrid leaf, 50X. A, midrib; B, small lateral vein; C, terminus of a veinlet. The laticiferous system has been removed, and only xylem remains.

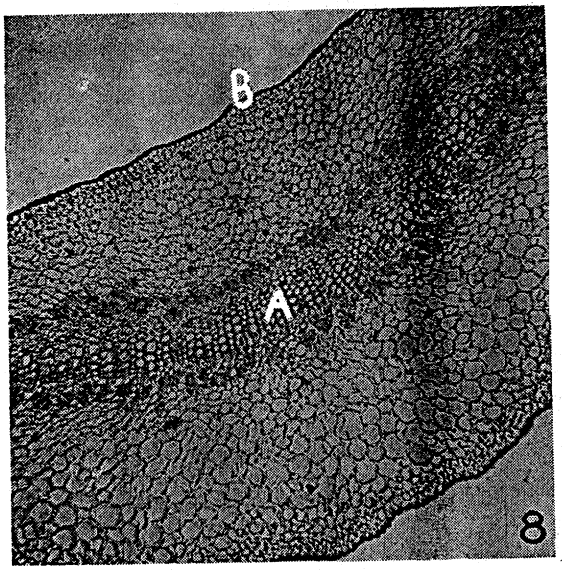


Fig. 8. Cryptostegia hybrid leaf, cross-section of midrib, acetone extracted, 50X. The stained areas surrounding the xylem (A) represent rubber in the laticiferous ducts. Approximately 100 ducts are associated with this vein. B, upper epidermis.

Fig. 9. Fresh, living, mature Cryptostegia hybrid leaf, cross-section, mounted in water, untreated, unstained, 460X. Rubber-bearing globules (A) surrounded by the darker, smaller chloroplasts in the palisade cells are shown. B, upper epidermis.

in a separate experiment on retted leaves (see Figures 6 and 7 for vein pattern) that the main vein, comprising the midrib and leaf stalk, contained more than half the xylem, by weight, of all the veins of the leaf. Since the globule-bearing cells are most numerous near the veins, extreme care was used in all cases in separating these cells from the veins under the dissecting microscope. The first lot of six leaves was separated into two fractions: 1(a) comprised the short leaf stalk, the main vein, and associated latex ducts, surrounded by epidermis; and 1(b) comprised the remainder of the leaf tissues. The second lot of six leaves was divided into three fractions: 2(a) was identical with 1(a) above; 2(b) comprised the largest lateral veins and associated ducts of each leaf; 2(c) comprised the remainder of the leaf tissues, including the smallest veins. The third lot of six leaves was not dissected and served as a control. All fractions were analyzed for total rubber. The results are shown in Table 1.

TABLE 1. ANALYSIS OF DISSECTED *Cryptostegia* HYBRID LEAF FRACTIONS

Leaf Fraction	Weight of Leaf Fraction, Grams*	Acetone Extract, %*	Benzene Extract, %*	Rubber	
				Weight, Grams	% of Total Rubber
1(a) Main vein ...	0.1772	7.53	6.58	0.0117	8.96
1(b) Leaf tissue minus main vein..	1.2480	14.72	9.53	0.1189	91.04
2(a) Main vein ...	0.1569	8.87	4.27	0.0067	5.34
2(b) Largest lateral veins	0.0378	13.95	5.02	0.0019	1.51
2(c) Remaining leaf tissue	0.9341	15.82	12.52	0.1169	93.15
3 undissected leaf...	1.0611	15.42	9.75	0.1035	100

* Moisture-free basis.

The high figures for the benzene extract are undoubtedly due to the removal of some of the non-rubber plant constituents by the acid, water, and alkali treatments before dissection. However the distribution of rubber, as shown in the last column of Table 1, is not affected by this pretreatment. The results indicate that 85 to 90% of the total rubber of these leaves occurred in individual cells, that only 10 to 15% existed in latex ducts, and that most of the latex rubber in the leaf was present in the stalk and midrib.

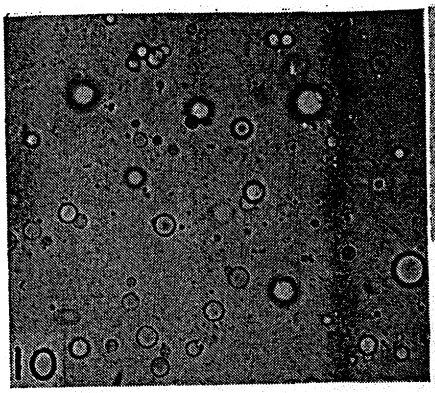


Fig. 10. Rubber-bearing globules isolated from Cryptostegia hybrid leaf chlorenchyma, 460X. Most of these globules are considerably larger than the rubber particles of the latex. Many of these globules are not in focus.

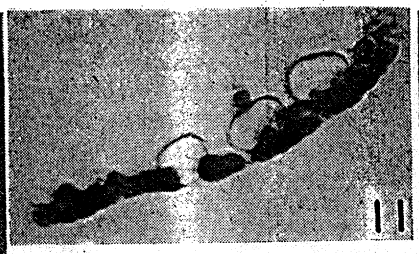


Fig. 11. Unstained palisade protoplast from retted Cryptostegia hybrid leaf, 860X. Three rubber-bearing globules partly surrounded by chloroplasts and protoplasm are evident. The cell was removed through the activity of microorganisms.

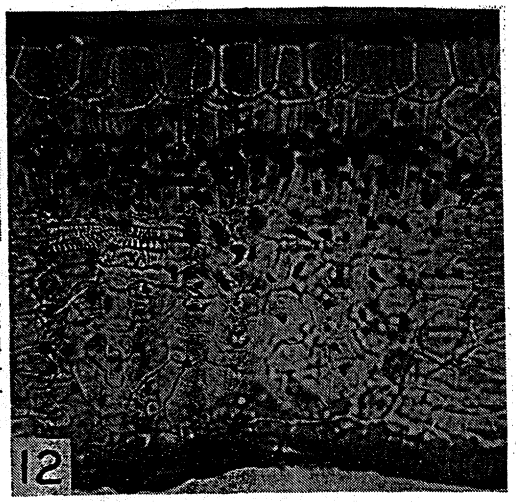


Fig. 12. Cryptostegia hybrid leaf, cross-section, 215X. The section was bleached with Javelle water, extracted with acetone for 48 hours, and stained with Calco Oil Blue NA. The stained bodies in the chlorenchyma are rubber.

Additional proof that rubber occurs in individual *Cryptostegia* leaf cells was furnished by isolating the leaf-cell globules and subjecting the isolated product to chemical analysis and physical tests. The isolation was accomplished by means of a process developed by Dr. Naghski and associates[14] for the recovery of rubber from *Cryptostegia* leaves. Special precautions were taken to assure the complete separation of the cell globules from the latex rubber.

Fresh, mature leaves of *Cryptostegia* hybrid were selected for the experiment. A representative leaf examined microscopically showed the presence of globules in the cells of the mesophyll chlorenchyma in great abundance (Figure 9). The rubber in the latex ducts was completely coagulated, as evidenced by its lack of Brownian movement and its elastic elongation. The leaves were retted anaerobically for four days with *Clostridium roseum* in a medium containing mineral salt. The vat contents were then stirred mechanically and finally filtered through a layer of cheesecloth. The filtrate was allowed to settle overnight, after a small amount of AgeRite was added to inhibit oxidation.

Microscopic examination of the unfilterable residue showed that it consisted of woody vein fibers, epidermis, latex ducts, strands of coagulated latex rubber, and a small quantity of cell fragments consisting of protoplasts with embedded globules. The settled layer from the filtrate consisted principally of chlorenchyma protoplasts with embedded globules, that is, essentially the original individual cell contents without the cell walls (Figure 11). No evidence of latex ducts or coagulated latex rubber was seen in this settled layer. Coagulated latex rubber may be recognized by its color, form, and granular structure. Some protoplasts with embedded globules and traces of latex duct fragments were found in suspension in the supernatant liquor.

The filtrate was centrifuged to sediment the protoplasts, and the supernatant liquid containing the traces of suspended latex duct fragments was removed by decanting. Separation of globules from the latex duct rubber was now regarded as complete. Two per cent. NaOH solution was then added to the moist sedimented fraction, and the mixture was boiled. As the boiling point was reached, the protoplasts began to disintegrate, freeing the globules (Figure 10). Then the suspension was centrifuged; this action produced on the surface a yellowish film composed of globules of various sizes, which spontaneously coalesced as the film dried. No evidence of protoplasts or coagulated latex rubber was found in this film. The dried film was yellowish, soft, tacky, and elastic and showed noticeable snap when stretched and released. The results of chemical analysis are shown in Table 2. Rubber was determined by precipitating the rubber as the tetrabromide.

It is pointed out that 7.91% of the crude product was nonrubber, benzene-soluble, acetone-insoluble material of unknown composition. The presence of rubber hydrocarbon in the acetone extract, as indicated by the analysis, might be expected if low-molecular weight rubber is present in the isolated product. Its presence in this extract was shown by precipitating the hydrocarbon gravimetrically as rubber "tetrabromide" and establishing the identity of the precipitate by a bromine analysis. Spectrophotometric examination of the benzene solution of the isolated product indicated the presence of pheophytin and carotene.

[14] J. Naghski, J. W. White, Jr., S. R. Hoover, and J. J. Willaman, unpublished report at the Eastern Regional Research Laboratory.

[15] T. J. Dietz, J. W. White, Jr., J. Naghski, and S. R. Hoover, unpublished report at the Eastern Regional Research Laboratory.

Table 2. Chemical Analysis of Fresh Mature *Cryptostegia* Hybrid Leaves (Starting Material) and the Globules Isolated from the Leaf Cells

	Original Leaves	Isolated Cell Globules
Wet weight, grams	3350	11.44
Dry weight, grams	450	8.66
Moisture, %	86.50	24.32
Acetone solubles by direct extraction, %*	9.39	29.56
Rubber hydrocarbon in acetone solubles, %*	..	3.75
Acetone solubles, non-rubber, %*	..	25.81
Acetone and benzene insolubles, %*	..	1.31
Total rubber hydrocarbon, %*	3.53	64.97
Non-rubber benzene-soluble acetone-insolubles, by difference, %*	..	7.91

* Moisture-free basis.

About 8.5 grams of the isolated cell globules, after being dried to about 16% moisture, were compounded according to a modified A.C.S. formula containing 50 parts of channel black. The vulcanizate had a tensile strength of 1200 to 1300 lbs./sq. in. and an ultimate elongation of 500%. These data do not necessarily indicate the quality of the rubber in *Cryptostegia* chlorenchyma cells since only one small sample was tested under a limited set of conditions. A more complete evaluation of the character and properties of the leaf-cell rubber will be published elsewhere.[15]

Final proof of the existence of rubber in the isolated cell globules was furnished by X-ray diffraction studies. At 320% elongation slight arcing of the innermost amorphous ring became evident. At 450% elongation eight crystalline reflections became visible. The interplanar spacings determined from these discrete reflections agree, within the limits of experimental error, with those of stretched *Hevea* rubber, indicating the presence of a *cis*-polyisoprene molecular structure.

Discussion

Microscopic observations of the rubber-bearing leaf cells during extraction experiments reveal the reasons why the rubber in these cells is not readily isolated and permit observation of the effects of various treatments. When untreated leaves are ball-milled, a moderate proportion of the cell walls are fractured. Although some of the rubber-bearing globules are liberated from the cell walls, they are not necessarily freed from the protoplasts, which prevent them from agglomerating into larger masses. Upon continued milling, however, the laticiferous duct rubber, supplemented by a fraction of impure globules, agglomerates into small masses or "worms." Usually these crude rubber masses

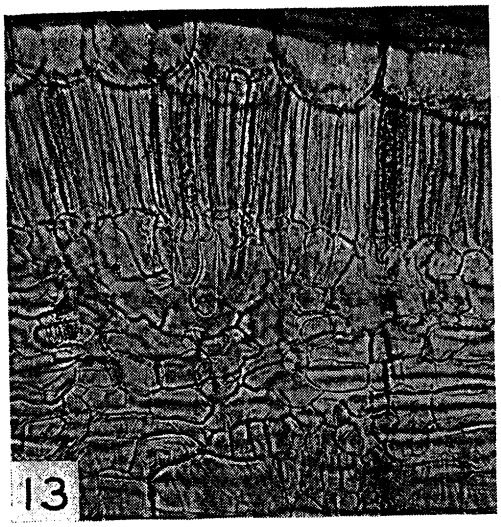
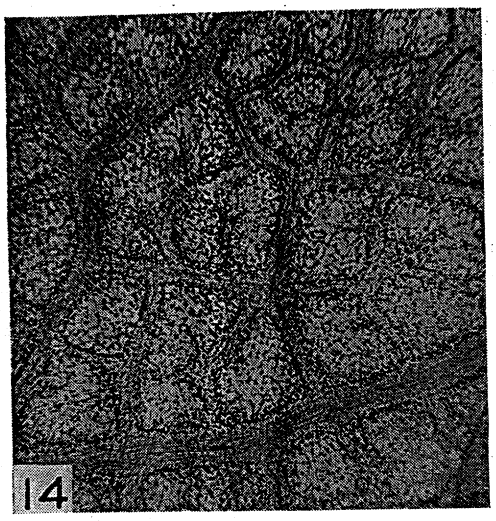


Fig. 13. Cryptostegia hybrid leaf, cross-section, 200X. The section was bleached with Javelle water, extracted with ether for 24 hours, and stained with Calco Oil Blue NA. No rubber remained in the section after ether extraction.

(Right)
Fig. 14. Cryptostegia hybrid leaf, longitudinal section of spongy mesophyll, treated with Javelle water, stained with Calco Oil Blue NA, 50X. Small, stained, rubber-bearing bodies of the chlorenchyma, most numerous in areas adjacent to the veins, are visible. The branching network of the background represents the fraction of the lower epidermis made visible by removal of the veins.



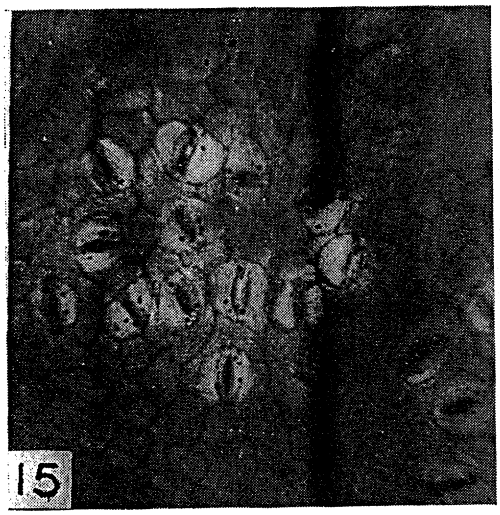


Fig. 15. Cryptostegia hybrid leaf, lower epidermis, treated with Javelle water, stained with Calco Oil Blue NA, 215X. The dark bodies within the guard cells aparently are rubber bearing.

are so highly contaminated with cellulosic and proteinaceous leaf matter that they sink in the flotation tanks and are therefore not readily separable from the non-rubber leaf debris. Only the relatively pure rubber masses float and are recovered. After a milling period of three or four hours the major portion of the leaf rubber is still found as scattered microscopic particles (globules), occluded by either the protoplasmic layers or the cell wall, or both. Pretreatment of the leaves, such as autoclaving with 2% sodium hydroxide or 2% sulphuric acid, although effective in reducing the total non-rubber leaf solids, does not by itself or in combination with milling result in the complete removal of the cellular barriers to rubber recovery. Greater success in rubber recovery accrues from the complete removal of the cell walls by retting followed by alkali dispersal of the occluding protoplasmic layers.[14]

The failure of many extraction experiments based on the assumption that all the leaf rubber occurs as latex in a duct system is readily explained by the anatomical findings. It is apparent that only a small quantity of rubber can be bled or leached from fresh leaves into latex-stabilizing solutions, since only 10 to 15% of the rubber occurs in a duct system. Likewise, attempts to obtain high recoveries by crushing or pressing rubber latex from the leaves are unsuccessful.[7, 8] Even when fresh leaves are rather finely macerated under a latex-stabilizing solution by a Buffalo or similar cutter, but little latex (always less than 10% of the total leaf rubber) is liberated into the solution. Most of the rubber remains unrecoverable as globules, occluded primarily by the protoplasts and, to a lesser extent, by cell walls.

The unusual disposition of rubber in *Cryptostegia* leaf is largely responsible for the difficulties encountered in developing an analytical method for the determination of rubber in the leaf. If ground leaves are extracted with acetone for 20 hours and then with benzene for 24 hours, a large portion of the rubber remains unextracted in the residue. No better results are obtained if the acetone-benzene extraction is preceded by a cold-water leach of the leaves. Microscopic examination of the extracted residue shows that most of the rubber has been removed from the laticiferous ducts, but that appreciable quantities remain in the unruptured chlorenchyma cells. However pretreatment of the leaf, such as autoclaving with dilute oxalic acid,[13] which alters the permeability or destroys the structure of the protoplasmic and pectinaceous layers surrounding the cell rubber, permits the complete extraction of resins and rubber. If such an extraction is followed microscopically, it is seen that, contrary to results with leaves not pretreated, the cell rubber is removed at a rate considerably faster than that for the duct rubber. Complete extraction is accomplished also after pretreatment with dilute sulphuric acid[16] although the extracted rubber is highly contaminated with non-rubber constituents.

Summary

Rubber occurs in *Cryptostegia* both in a laticiferous duct system and in individual leaf chlorenchyma cells. The ducts occur principally in the stems, but extend into the leaves, paralleling the vein system. Dissection and chemical analysis indicate that 85 to 90% of the rubber in a mature *Cryptostegia* hybrid leaf exists in the chlorenchyma cells and the remaining 10 to 15% in the ducts.

That the globules occurring in the chlorenchyma cells contain rubber is indicated by staining, solubility, micrurgical, dissection, and analytical tests on leaf sections; it is proved beyond question by chemical, physical, and X-ray analyses of globules isolated in quantity. These globules contain approximately 65% rubber hydrocarbon, apparently of low molecular weight.

The occurrence of the major part of the rubber in the leaf as globules entrapped in protoplasts and surrounded by cell walls accounts for the difficulties encountered in extracting rubber from *Cryptostegia*. These findings have been valuable in indicating new lines of approach to the problem of extracting rubber from *Cryptostegia* and in providing new information regarding the formation and storage of rubber in this plant.

We gratefully acknowledge the cooperation and suggestions of the members of this Laboratory who worked on the Emergency Rubber Project. Special credit is due Albert Kelner, who first observed the cell globules and suspected that they contained rubber.

[16] D. Spence and M. J. Caldwell, *Ind. Eng. Chem., (Anal. Ed.)*, 5, 371 (1933).